1973

Tissue culture of Camptotheca acuminata decaisne (Nyssaceae)

Ron Charles Cooke
University of the Pacific

TISSUE CULTURE OF *CAMPTOTHECA ACUMINATA* DECAISNE (NYSSACEAE)

A Thesis
Presented to
the Graduate Faculty
University of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Masters of Science

---

by
Ron Charles Cooke

April 1973

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

Camptotheca acuminata Decaisne (Nyssaceae) is endemic to China. The plant was brought to the United States only on a few occasions and thus can be considered rare in this country. A search of the plant kingdom for species that produced anticancer substances revealed that ethanolic extracts of *Camptotheca acuminata* had such a property. The active constituent was identified eventually. Further testing and possible marketing of the active principle, an alkaloid, (camptothecin) depended on a combination of factors: obtaining the plant from China, organization of a mass planting program in the United States or chemical synthesis of the drug.

Due to the dissention between China and United States during this period, (1950-1960's) the first alternative was ruled out. Planting programs were organized but the plant needs a relatively long time to mature. Attempts to synthesize camptothecin were started but success was not immediate and there was no guarantee of ever achieving synthesis.

As an alternative to these methods, the author of this research proposed an *in vitro* system of obtaining the drug. The plant would be grown in culture and the active principle extracted.

The complete story of camptothecin, the rationale for *in vitro* culture in general and the methods and results of this project are given in the following chapters.

Chapter 2

A REVIEW OF LITERATURE AND PROCEDURES

Introduction of the Plant

The genus Camptotheca consists of a single species indigenous to the interior of The Peoples Republic of China. It grows to heights of sixty feet along river beds and other wet areas. It was introduced to the United States of America on four separate occasions (Perdue 1968).

In 1911, seed was collected by E.H. Wilson, Mt. Omei, Szechwan Province and sown at the Arnold Arboretum in February 1911. Plants were supplied to the United States Department of Agriculture (USDA) in November 1912 and accessioned as Plant Inventory (P.I.) Number 34534.

In 1927, plants were obtained, probably from the Nanking Botanical Garden, by W.T. Swingle and received by the USDA in January 1927 and accessioned as P.I. 71179.

In 1934, seed was collected by A.N. Steward in Chang An, Yung Hsien and planted at the USDA Plant Introduction Station, Glenn Dale, Maryland in June of 1934 and accessioned as P.I. 132293.

In 1949, seeds were obtained from Lu Shan Arboretum and Botanical Garden by William Hagen, nurseryman in Arcadia, California and distributed in the West Coast states.

The Maryland weather was too harsh for the plants grown from seed introduced in 1934. Several grown in greenhouses were shipped to the Chico, California USDA Plant Introduction Station

(Chico USDA Station). Two trees survive today as well established seed bearing trees (numbers K5-6, K6-6). Initially it was thought that the rapid growing Camptotheca acuminata (C. acuminata) might prove to be valuable shade trees, but their development showed they would be only of a poor quality and of lesser value than other easily grown species. Therefore they had little apparent value and were permitted to remain in place only because the space was not needed for other plantings.

Discovery of the Anticancer Property

The first chemical studies of the plant were reported by Wall and coworkers (1966) and the results revealed no significant test for saponins, alkaloids or flavonoids. This work was part of the USDA search for plant species that produce precursors to cortisone. The USDA Plant Introduction Stations in California, Florida and Georgia which keep species that have been introduced into the United States from foreign countries participated in this search.

C. acuminata was one of many plants supplied by the Chico USDA Station. Although the plant extract proved negative for cortisone precursors it was bottled and stored for any future use that might arise.

In 1957 the Cancer Chemotherapy National Service Center (CCNSC) of the National Cancer Institute (NCI) began screening plants for substances with antitumor activity. Not only were new plants investigated but the old extracts from the cortisone screening were tested as well. The C. acuminata extracts exhibited preliminary experimental activity against the adenocarcinoma 755.[1]

---

[1]Unpublished screening data, CCNSC, NCI.

Subsequent NCI data indicated that the extract had activity against the Lymphoid Leukemia L-1210 test system (L-1210). Perdue et al. (1968) pointed out that activity against L-1210 in laboratory mice is a valuable test as the system appears highly predictive of anticancer activity in humans. This ascitic form of L-1210 was selected for use in the CCNSC screening program in 1955. The choice was based on its ability to select drugs that may prove valuable to man. The L-1210 is a reproducable tumor system in which variations in response are more likely due to chemical changes in the test material rather than to tumor variations.

Testing of plant extracts for activity in the L-1210 system is controlled and evaluated under CCNSC protocol (CCNSC, 1962). Preliminary testing is done in two stages. Six mice are used in each chemical test group (T) and six mice in a control group (C) without chemical agent. The survival in days (mean value) of the T group is compared to that of the control. The extract passes the stage-I examination if non-toxic and $T/C \geq 125$ percent. When the test is repeated stage-II is passed if the product of T/C when the test is repeated and the stage-I T/C is ≥ 1.56 (when expressed as a decimal). Finally the activity is confirmed by testing two new extracts from the same plant sample in two dose-response experiments. In these tests the extracts are tested at $\frac{1}{4}X$, $\frac{1}{2}X$, 1X and 2X the dose which originally passed stage-I and at least one test in each of the two dose-response experiments must yield $T/C \geq 125$ percent.

Although the extracts from the Chico C. acuminata had been made in 1951 for the cortisone program and then stored for 7 years, in 1958, in the L-1210 test a dose of 500 mg/Kg gave a T/C of 154 percent (Perdue et al., 1968). There was not enough of the original extract left to continue testing.

Leaves, twigs and fruit were collected in 1961 from the Chico USDA Station to continue the testing. It was found that the leaf extract was negative but the twig and fruit extracts were positive. It was also noted that an aqueous extract of fruit showed no activity against L-1210 but an ethanolic fruit extract was active (Perdue et al., 1968).

Data pertaining to the initial testing of C. acuminata are presented in Table 1 (Perdue et al., 1968).

Table 1. Screening Results of Ethanolic Extracts of Camptotheca acuminata fruit

Test	Test Date mo.-year	Dose mg/Kg	T/C %
Stage-I	2-62	400	133
Stage-II	3-62	400	147
Dose-response 1	4-62	800	145
	4-62	400	160
	4-62	200	133
	4-62	100	117
Dose-response 2	6-62	350	144
	6-62	175	135
	6-62	87.5	124
	6-62	44	110

Isolation and Structure Identification

As the interest in the C. acuminata extracts grew, work was begun to identify the substance or substances responsible for the anti-cancer activity. This work was carried out by Dr. M.E. Wall and coworkers

(1966) at the Research Triangle Institute, Durham, North Carolina. Extraction consisted of continuous hot hexane-heptane extraction of the dried powdered plant material followed by hot continuous extraction with 95 percent ethanol. The ethanolic extract was concentrated and then partitioned between water and chloroform. Silica gel chromatography of the methanol-insoluable material from the chloroform phase, followed by crystallization from methanol-acetonitrile yielded the new alkaloid camptothecin (Figure 1). The structure was established using a combination of chemical and physical techniques. Camptothecin is an unusual alkaloid which does not react with typical alkaloidal reagents nor does it form the common alkaloidal salts. This probably explains why it was not assigned the classical alkaloid designation "-ine" i.e. camptothecine.

Camptothecin does not form stable salts with acids and on treatment with base (NaOH) the lactone shows unusual reactivity by immediate conversion to the sodium salt (Figure 2). This sodium salt has the same order of antileukemic activity as camptothecin (Wall et al., 1966).

Further fractionation led to the isolation of two related compounds hydroxycamptothecin and methoxycamptothecin (Wani and Wall, 1969) (Figure 3). Both have activity against L-1210 but neither of these related compounds nor any synthetic derivative to-date have shown greater experimental antitumor activity than the parent compound (Wagner and Horhammer, 1971).

The physical data for camptothecin, hydroxycamptothecin and methoxycamptothecin are given on page 8.

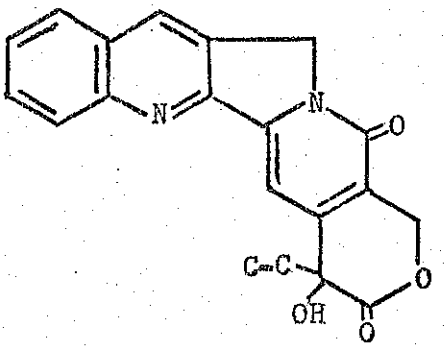


Figure 1. Structure of Camptothecin

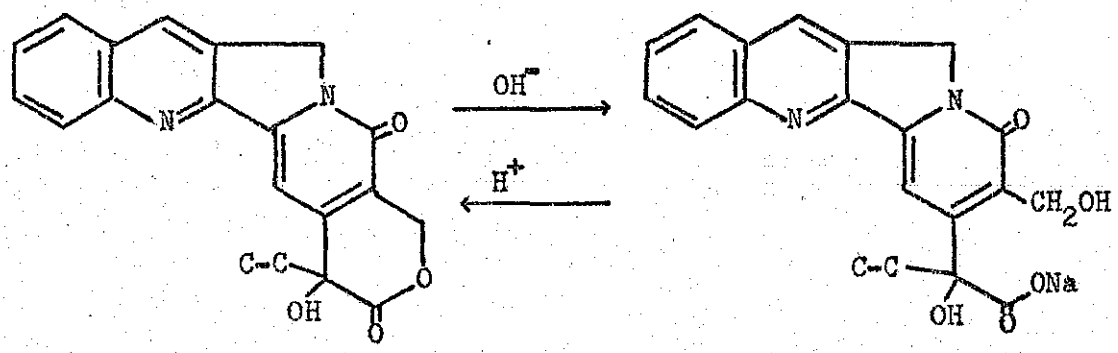
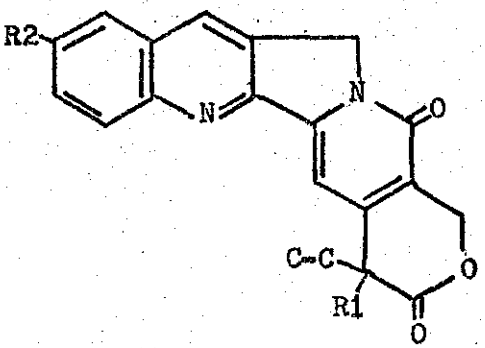


Figure 2. Interconversion of Camptothecin and the Sodium Salt



Camptothecin R_1=OH, R_2=H

Hydroxycamptothecin R_1=R_2=OH

Methoxycamptothecin R_1=OH, R_2=OCH_3

Figure 3. Structure of Camptothecin and Derivatives

Camptothecin

Molecular Weight 348
m.p. 264-267 dec. (Kofler Microstage)
UV_{max} 220 nm (ε 37,320), 254 (29,320), 290 (4,980), 370 (19,900) in 95 percent ethanol
ir (KBr) 3440 (hydroxy), 1760-1745 (lactone), 1660 (lactam) and 1610, 1585 cm^{-1} (aromatic)

Hydroxycamptothecin

Molecular Weight 364
m.p. 268-270
UV_{max} 222 nm (ε 50,300), 267 (27,400), 330 (12,100), 382 (28,000) in methanol
ir (KBr) 3480 (hydroxy), 1740-1755 cm^{-1} (lactone)

Methoxycamptothecin

Molecular Weight 380
m.p. 254-255
UV_{max} 220 nm (ε 49,900), 264 (29,800), 293 (5,700) 312 (8,600), 328 (12,000), 365 sh (27,500), 379 (31,500) in methanol
ir (KBr) 3330 (hydroxy), 1750 cm^{-1} (lactone)

Clinical Studies on Camptothecin

Clinical studies with the sodium salt of camptothecin were begun at the Baltimore Cancer Research Center in January 1969 (Gottlieb et al., 1970). The alkaloid was given to 18 adults (8 women and 10 men with a mean age of 49.7 years) during a clinical trial to establish dose. Sixteen patients with various advanced solid tumors received 35 intravenous (IV) injections of 0.5-10.0 mg/kg of body weight at intervals of 2-4 weeks. Two additional patients received 5 injections of 2 mg/kg at intervals of 3 days. Partial remissions (decreases) of greater than 50 percent tumor mass were noted in 5 patients and evidence of objective response or 25-50 percent remission was noted in 6 patients. Three patients remained unchanged or an increase in tumor mass was noted and treatment was discontinued for three patients due to insufficient hydration during

therapy, which led to a severe sterile hemorrhagic cystitis. The treatment was accompanied by improvement both in nutritional and in performance status of patients. Toxicity was generally acceptable, consisting of alopecia, mild gastrointestinal upsets, hemorrhagic cystitis and dose-limiting myelosupression which in some cases necessitated platelet transfusion. Four patients died 7-17 days after the drug was administered. The deaths did not appear to be directly drug related although all four patients had impaired excretion and subsequent profound hematopoietic toxicity.

Other studies by this group revealed that a high percentage of camptothecin was protein bound after injection. A mean of 17.4 percent of unchanged drug was excreted in the urine in the first 48 hours after administration. Thin-layer chromatography (TLC) evaluation showed no fluorescent urinary metabolites and no drug was found in various body organs or the tumor.

The method used to assay for camptothecin level was that of Hart et al. (1969), a fluorimetric analysis sensitive to 0.005 μg/ml for physiological fluids.

Although this preliminary evaluation seemed promising, Wood (1971) has stated that in view of the toxicity of sodium camptothecin in recent clinical testing, the compound would have limited use. Thus efforts would be directed toward other structural analogs of camptothecin, notably hydroxycamptothecin and methoxycamptothecin.

The Problem of Obtaining Camptothecin

It became evident, after learning of the potential use of camptothecin, that the two trees at the Chico USDA Station could not

provide enough raw material to complete the research unless they were both sacrificed. It was not possible to obtain C. acuminata from China. The two trees in Chico, as far as was known were the only two in the United States or territories friendly to the United States and were mature and seed bearing and could not be sacrificed.

A search for C. acuminata along the west coast, through the joint efforts of botanists, horticulturists, colleges, universities, nurseries and parks led to the discovery of 30 small trees in central and southern California. These trees were removed as the material was needed for investigation.

In 1963, USDA horticulturist R.L. Smith at the Chico USDA Station succeeded in germinating seeds from the 1963 crop. Approximately 300 seedlings were transplanted to the field and by 1964 they were 45 cm tall. Investigations were made as to the potency of young plants. It was found that they were active in the L-1210 test system and it appeared camptothecin yield was not related to tree age. This implied that maximum camptothecin production would be at the stage when the tree produced maximum yield of dry matter (Perdue et al., 1968). A supply of natural material was now available but required germination of seeds, planting of seedlings under glass, transporting the seedlings outside and finally harvesting the plant, grinding and then extraction and purification of the crude material. The tree of course would be destroyed in this process.

An alternative to the destruction was suggested by Smith (1971) who proposed harvesting the leaves only of C. acuminata. However it was noted that the leaves had the lowest concentration of all plant parts (Perdue et al., 1968).

Chemical Synthesis of Camptothecin

The reports of the promising antitumor properties of camptothecin and the problem involved in obtaining plant material sufficient for extraction led to attempts at chemical synthesis of camptothecin. Total synthesis was first reported in 1971 (Stork and Schultz, 1971; Volkmann et al., 1971). Another synthesis of this compound was recently reported by Wani et al., (1972). In all three cases the product was a racemic (dl) mixture and in small yields. To-date there are no reports of antitumor testing of the synthetic dl-camptothecin.

Mode of Action of Camptothecin

Many researchers have investigated the mode of action of camptothecin (Bosmann, 1970; Horwitz et al., 1970; Kessel, 1971 a,b.; Moore et al., 1970). In general, most agree that ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein synthesis is inhibited by camptothecin or sodium camptothecin. The inhibition seems to be concentration dependent and is reversible if the drug is washed away from the test system. In general, the test system involved the use of a radioactive purine or pyrimidine or amino acid. The incorporation of radioactive material into DNA, RNA or protein was measured and plotted with respect to drug concentration. The mechanism of action is still questionable but it is proposed that camptothecin acts at the level of the DNA and RNA polymerase.

Plants-General Considerations

Green plants, unlike animals, do not require a source of complex foods and vitamins daily to survive. Plants are autotrophic-capable of synthesizing organic substrates from inorganic starting materials using

light from the sun as energy. The green leaves with their photosynthetic ability provide organic materials for the metabolic and synthetic activities of the whole plant. Plants could be grown in water without soil if the proper minerals were added and if the plant was provided with sunlight and air. Water cultures of higher plants (hydroponics) were routinely carried out in the late 1800's using an aqueous solution of calcium nitrate, magnesium sulfate, potassium phosphate and ferrous sulfate (Torrey, 1967). Thus came the belief that these added elements plus those provided in the atmosphere were the basic essential elements. These elements have been arranged to give a mnemonic phrase C HOPK'NS CaFe Mg or "See Hopkins Cafe, mighty good". These are the essential macronutrients in which the concentration required for plant growth is 100-2,000 parts per million (ppm)[2].

To be essential, an element must be necessary for normal growth or reproduction, neither of which can occur in its complete absence. The element requirement must be specific and cannot be replaced by substituting another element. In addition the element must be acting directly on the plant and not simply causing some other element to be more available or counteracting a toxic element.

In the early days of hydroponics, chemicals were not as pure as they are today and contained many other elements as impurities. If a plant were grown today in the original hydroponic solution consisting of glass distilled water and analytical grade chemicals, it would show certain deficiency symptoms from a lack of some elements required

---

[2]Iron actually needs to be provided only in a few ppm and should thus be characterized as a micronutrient.

in minute quantities only.

A group of trace elements has been added to the essential requirements, these are the micronutrients, which are necessary in quantities less than 100 ppm. These include the elements iron, molybdenum, boron, copper, zinc and chlorine.

Although most higher plants are thought to require only those elements mentioned above, certain species may require others such as sodium, silicon, aluminum and cobalt.

Listed below are generalized functions associated with each of the essential elements. In the intact plant, defiency symptoms are usually found in response to a lack of any essential element. These functions were abstracted from Salisbury and Ross (1969).

Carbon, hydrogen and oxygen are brought into the plant by absorption of water and carbon dioxide. These three elements form the major part of hundreds of organic materials synthesized in the plant. Carbon and oxygen make up the bulk of the dry weight of the plant (45 percent each) and hydrogen is next with 6 percent.

Nitrogen is essential to plants because it is part of a large number of necessary organic compounds including amino acids, proteins, coenzymes, nucleic acids, chlorophyll and plant hormones. It is also required for alkaloid synthesis. Plants cannot utilize elemental nitrogen which represents about 78 percent of the atmosphere. Nitrogen is utilized as nitrate ion and ammonium ion.

Phosphorous is absorbed by plants as the monovalent or divalent phosphate ion. Molecules containing phosphate include nucleotides, nucleic acids and phospholipids.

Potassium is absorbed as the monovalent ion. Potassium does not

form a structural part of any molecule inside plant cells but it is required for growth and development. It seems to be required for activation of certain enzymatic reactions notably to do with protein synthesis.

Sulfur is absorbed as the sulfate ion and incorporated into molecules such as sulfur containing amino acids, coenzyme A and the vitamins thiamine and biotin.

Magnesium is an essential part of the chlorophyll molecule and also serves to activate certain enzymatic reactions.

Calcium is a constituent of the middle lamella of the cell wall, where it occurs as calcium pectate. Calcium pectate is thought to cement adjacent primary cell walls together. Calcium is also required to maintain membrane structure and differential permeability. Calcium is an essential activator of certain enzymes.

Iron is necessary in the synthesis of the chlorophyll molecule. It is believed that it activates one or more enzymatic reactions but most of these have not yet been thoroughly investigated. Iron is present in the cytochromes and is an activator for certain enzymes.

Chlorine is absorbed as the chloride ion and usually does not become a structural part of any organic molecule. It acts as an enzyme activator in the process of photosynthesis.

Manganese is absorbed as the divalent manganous ion. Lack of manganese causes a disorganization of the membranes of the chloroplast suggesting a structural role in the chloroplast membrane. Manganese also activates several enzymes.

Boron is required for normal carbohydrate transport in the phloem but the mechanism is not understood. It is absorbed as the trivalent

borate ion.

Zinc is a required activator for many enzymes and is needed for the synthesis of indoleacetic acid, a plant hormone.

Copper is required for certain enzymatic reactions and is a part of the molecule plastocyanin, a compound involved in the photosynthetic electron transfer chain.

Molybdenum is required in the least concentration of all the essential elements and is absorbed as a molybdate salt. Its best known function is as an electron carrier when present in certain enzymes necessary to convert nitrate to ammonia ions.

A list of the total essential elements and the concentrations required for adequate plant growth are listed in Table 2.

Plant Culture

Three main lines of development can be traced under the general category of plant culture or plant tissue culture. These three have been concerned with: (1) the culture of isolated plant organs (isolated roots and to a lesser extent, shoot apices, leaf primordia, flower structures and immature embryos); (2) the growth of tissues or callus cultures (proliferating undifferentiated cells); (3) the growth of mixed suspensions of cells and small cell groups.

An objective stated by Haberlandt (1902) concerned the desirability of culturing the isolated vegetative cells of higher plants in order to demonstrate experimentally the suspected totipotency of all living higher plant cells and the possibility of directing experimentally the process of cellular differentiation. In addition, the cell's nutritional, physical and growth factor requirements would be shown. Haberlandt was faced with a number of difficulties- plant cells are not

Table 2. Elements Essential for Most Higher Plants and Internal Concentrations Considered Adequate[a]

Element	Form available to plant[b]	Concentration in dry tissue (ppm)	(percent)
Molybdenum	$MoO_4^{=}$	0.1	0.00001
Copper	$\underline{Cu^{++}}$, Cu^{+}	6	0.0006
Zinc	Zn^{++}	20	0.002
Manganese	Mn^{++}	50	0.005
Iron	Fe^{++}, Fe^{+++}	100	0.01
Boron	BoO_3^{-3}	20	0.002
Chlorine	Cl^{-}	100	0.01
Sulfur	$SO_4^{=}$	1000	0.1
Phosphorous	$\underline{H_2PO_4^{-}}$, $HPO_4^{=}$	2000	0.2
Magnesium	Mg^{++}	2000	0.2
Calcium	Ca^{++}	5000	0.5
Potassium	K^{+}	10,000	1.0
Nitrogen	$\underline{NO_3^{-}}$, NH_4^{+}	15,000	1.5
Oxygen	O_2, H_2O	450,000	45
Carbon	CO_2	450,000	45
Hydrogen	H_2O	60,000	6

[a]Modified after Salisbury and Ross (1969).

[b]The most common form is underlined.

bathed in a free, complete nutrient medium; the xylem contains the required inorganic ions but lacks organic compounds; the phloem contains organic compounds but is in direct contact with only a small percentage of the total plant cells. The majority of cells must therefore obtain the requisite nutrients by cellular diffusion. Liquid endosperm (the nutritive substance formed within the embryo sac providing food for its development) is the only natural nutrient that could be used for cellular cultivation but in general is present in only minute quantities.

Other problems he encountered included the plant cell wall which prevents the cell from engulfing food and also prevents adhesion to a substrate. Additionally, plant growth occurs in only a few specialized regions such as roots, shoots and cambium. The remainder of the plant is left in a quiescent condition where cell division rarely occurs.

Animal cells do not have the culture problems associated with plant cells, namely: animal cells are bathed in a fairly complete nutrient media (blood, lymph or intersticial fluids). These fluids can be removed for *in vitro* use. The mobile animal cell membrane can adhere to substrates, engulf food and aid in movement. In addition cell division occurs in all parts of the animal. As a result, cell culture was initially restricted to the animal kingdom and by 1907 reproducable techniques for *in vitro* animal cell cultures were available (Harrison, 1907).

Kotte, (1922) a student of Haberlandt and, independently, Robbins (1922) in the United States, succeeded in obtaining considerable, but not indefinite growth of *Zea* root tips *in vitro*. It was found that on these initial media (*e.g.* inorganic ions, 1 percent dextrose and a meat

extract) the growth was limited and with each subculture there was a marked and progressive decrease in growth rate.

In 1932 P.R. White began work on root culture. He systematically varied such factors as light, temperature, aeration, volume, pH, inorganic and organic constituents. Finally in 1934 he succeeded in developing a culture medium that would support continued growth of tomato roots without loss of growth rate. The major change was a substitution of sucrose for dextrose. The lateral root tips which developed *in vitro* could be subcultured, thus from a single root tip, a clone of excised roots could be established (White, 1934).

The same strain of roots started at that time was, as of 1963, still viable after 29 years and more than 1,600 passages (White, 1963).

At the same time as White succeeded in culturing root tips, Gautheret (1934) in Paris, succeeded in the cultivation of cambial tissues on a solidified medium. Independently Nobecourt (1937) in Grenoble and White (1939) also succeeded in cultivation of cambial tissues for prolonged periods of time. The work of these three represents the first true plant tissue culture in the sense of culture of unorganized cells.

Work with callus tissue indicated that there was a lower limit to the size of tissue fragments that could be subcultured on solid media. below this size limit growth was uncertain and if growth did appear, it was only after an extremely long lag period (Willmer, 1966).

Muir *et al.* (1954) reported the growth of liquid cultures containing single cells and small cell groups. These shake cultures resulted from transferring fragments of callus tissue (*Tagetes erecta* and *Nicotiana tabacum*) from a solidified medium to a liquid medium.

Rinert (1956) reported evidence of cell division in a similar suspension of single cells and cell clumps of Picea glauca. Muir et al. (1954) succeeded in getting closer to Haberlandt's dream of culturing single, isolated plant cells by isolating single cells from suspensions and placing these individually on the upper surface of small squares of filter paper whose lower surface was in contact with activly growing "nurse" callus on solid medium. Many of these single cells underwent cell division and a few continued to divide yielding small callus masses which could be transferred to solid medium to give a callus clone of single-cell origin.

Bergman (1960) took suspension cultures of Nicotiana tabacum and Phaseolus vulgaris with 90 percent of the filtrate containing single cells and spread them over the surface of a solidified agar medium in a petri plate. Individual cells could be observed and marked. After incubation, 20 percent of the cells underwent repeated cell division giving rise to small colonies which could be subcultured.

Prior to discussion of each type of plant culture a review of plant development or morphogenesis is necessary. The use of organ, tissue and cell culture has helped greatly in the better understanding of the three levels of developmental control viz. intracellular (genetic), intercellular and extracellular control.

As noted earlier, isolated plant cells can be grown in sterile culture to yield masses of callus tissue. Further work has shown that these masses, when placed on a special medium, can develop into an entire plant capable of reproduction (Skoog and Miller, 1957). This work carries with it the implication that each mature cell in the plant carries in its nucleus all the genetic material required to reproduce

the entire ontogeny of the plant. The genetic control of development depends upon the sequential activation of genes. This is the mechanism whereby the genetic information contained in DNA is transcribed by RNA synthesis. RNA templates then aid in the translation of information necessary for protein synthesis. This protein synthesis leads to specific enzyme activity and differentiation. It has been shown that differences occur in the messenger RNA (m-RNA) and protein of newly differentiating tissue as compared to undifferentiated tissue thus supporting the theory that new genes begin to function during differentiation (Salisbury and Ross, 1969).

Therefore, if it is assumed that cellular activities are controlled ultimately by gene action, the problem of development in multicellular organisms can be reduced to the understanding of the control mechanisms capable of switching genes on and off in an orderly sequence to produce the coordinated series of cellular events which make up the ontogeny of the individual (Torrey, 1967).

The orderly control of gene action is then a key question in the understanding of development. Work by Jacob and Monod (1961) with bacterial systems has shown a possible mechanism in which the control of enzyme synthesis is under the control of structural genes. Each structural gene codes for a particular m-RNA molecule, which in turn, in conjunction with ribosomes and transfer RNA (t-RNA) codes for the structure of a particular enzyme or polypeptide chain. These structural genes can form m-RNA only when an adjacent portion of DNA in the chromosome, called the operator gene, is in the functional state. As long as this operator gene is open all structural genes under the control of this operator can synthesize m-RNA. The position of the operator (open or closed)

depends on another type of gene known as a regulator gene. This gene acts negatively; that is, it closes the operator gene by inhibition with a repressor substance which in its active form is thought to combine with the operator region preventing initiation of m-RNA synthesis. The repressor substance can be acted upon by other molecules, namely, inducer or corepressor substances. An inducer substance causes the formation of m-RNA (and enzyme) by combining with the repressor and therefore preventing the repression of the operator. Enzyme repression occurs when a corepressor molecule aids in establishing the repressor substance in its active form and hence blocking the operator gene.

In the next section on the second level of plant developmental control an example of this type of regulation in plants will be proposed.

The hormonal control of development is provided by at least three groups of substances known to act as regulators of plant development. These three groups are the auxins, the gibberellins and the cytokinins. All groups have certain features in common: they are organic compounds of low molecular weight, active at very low concentrations and in accordance with the definition they are synthesized in one part of the plant and translocated to another part where they elicit profound controlling or regulatory effects. They have multiple effects involving cell division, cell enlargement and cell differentiation. They also interact in their control of development to such an extent that with any tissue response to an exogenously supplied regulator it must be assumed that the possibility exists that each of the other two types of regulators may be present endogenously and participate in the tissue response (Torrey, 1967).

The exact mechanism of action of the plant hormones is not clearly understood to-date but they probably act by controlling enzyme production

or enzyme activity or both. It is thought that they may act as inducer or corepressor molecules and thus affect the gene action through repressor substances. Support for this theory has been shown by Paleg (1960). It has been noted that the embryo of seeds of cereal grains is surrounded by nonliving endosperm. The endosperm is surrounded by a thin layer of living cells called the aleurone layer. When germination occurs in response to increased moisture content, the aleurone layer provides the hydrolytic enzymes that are necessary to digest the starch, proteins and RNA present in the endosperm. These digestion products are then used by the embryo during its early development. The enzymes required for this process include β-amylase already present in the aleurone layer; α-amylase synthesized in the aleurone, ribonuclease and proteases. If the embryo is removed from the seed and moisture added the aleurone cells do not produce enzymes. If gibberellic acid 3, a gibberelin was added in concentrations as low as 2×10^{-11} M the production of α-amylase and proleolytic enzymes was induced and the β-amylase already present was activated. This is the most clear cut example of plant hormone action upon gene activation. Other mechanisms have been speculated but require proof and Torrey (1967) has stated that it would be wrong to presuppose that all hormonal effects involve action at the level of genes. There is no evidence for such a supposition. But it is useful to realize at the outset that some hormonal effects do operate at this level.

Auxins are synthesized in the plant in the terminal buds of the shoot and in the youngest leaves. They move down the plant in a strictly polar fashion from the tip to the base. Auxins have a notable capacity to stimulate cell elongation. In addition they have a capacity to induce cell division as in the induction of lateral roots. Many commercial

preparations of auxin are used to induce roots from cuttings. They exhibit inhibitory action such as the inhibition of axillary buds by auxin production in the terminal bud. The most common natural auxin is indole-3-acetic acid (IAA). In addition some synthetic compounds possess some of the typical auxin activity. The synthetic auxins are commonly used in experimental plant work and plant tissue culture and include napthalene acetic acid (NAA), indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D).

Gibberellins are naturally-occurring plant hormones synthesized in the young leaves. Many of these closely related gibberellic acid (GA) compounds occur and are identified by a number e.g. GA-3. They all seem to possess similar physiological activity but differ quantitatively in the physiological response elicited. These substances have many effects when applied exogenously. The most striking is stem elongation where the primary cause is increase in cell length rather than increase in the number of cells.

Certain experiments had shown that degredation products, resulting from autoclaving DNA, would cause cell division of cells grown in culture (Jablonski and Skoog, 1954). A compound was synthesized from DNA and was identified as 6-furfurylamino purine (kinetin) which is a derivative of adenine (Miller et al., 1955). This compound was shown to stimulate cell division. It has not been shown that kinetin occurs naturally but other natural cytokinins do exist. An important characteristic of kinetin is that it is virtually ineffective in the absence of auxin (Audus, 1959).

In addition to their action in stimulating cell division the cytokinins, when applied exogenously, are known to delay protein and

chlorophyll breakdown in detached leaves. This may represent action as an antisenescence hormone in the intact plant (Salisbury and Ross, 1969). If the leaf is treated with kinetin while intact, not only does the breakdown of chlorophyll and protein decrease but nutrients are mobilized and directed toward the point of application.

Cytokinins have the ability of interacting with auxin to induce organ formation. Skoog and Miller (1957) have shown that pith cells from the stem of Nicotiana tabacum could be induced to form callus and/or roots and/or shoots or to show no growth at all simply varying the relative ratio of IAA to kinetin with all other factors remaining constant.

Genetic and hormonal controls reside within the plant and may be closely related. In addition to these internal controls plants develop in response to external and environmental stimuli. These responses are in general quite complex. Light and temperature are very important external stimuli. Temperature has an effect on reaction rates. Temperature can have an inductive effect such as formation of tubers or other underground storage products in response to cold temperatures. The effects of light depend on the intensity, quality (wavelength), and duration (Salisbury and Ross, 1969). The direct effect of light is photosynthesis. Many other effects can be seen only after a period of time. These effects include breaking of dormancy in certain seeds, stimulation of anthocyanin pigment production and bending of a plant towards a light source. Gravity is another environmental stimulus. A seed develops with the shoot up and the root down as a response to gravity. Other environmental stimuli are touch or injury.

The mechanism of action of most environmental stimuli is largely unknown but must be interpreted as acting through cellular responses.

These may affect hormone synthesis which will in turn act at the genetic level (Torrey, 1967).

Therefore, certain nutritional requirements and the many control levels have been discussed, including some proposed mechanisms of action. At the present time it is impossible to interpret and explain how the interaction of all these factors give rise to plant development (which can be extrapolated to plant tissue culture and plant tissue development) but it does give a rationale for many of the methods and chemicals used in plant culture.

Organ Culture

The degree to which one plant organ (root, leaf, stem and flower parts) is dependent on the plant as a whole is seen if the organ is removed and maintained separately. This culture of organs shows the degree of synthetic power residing in the organ. This procedure also shows that synthetic ability which does not reside with the organ and must be provided.

As was noted earlier, the first successful plant organ culture was of the isolated root tip. The root tip required all the nutrients, vitamins and minerals it had in the intact plant. The root is not photosynthetic and must be provided with an energy and carbon source such as sucrose. The root tip culture also required all the minerals the root needed when in contact with the soil. These required minerals are the essential elements (Table 2, p. 16). In addition most species of roots require one or more of the B-vitamins, usually thiamine and pyridoxine and/or niacin. The root apparently lacks the enzymes necessary to synthesize the vitamins independently and in the intact plant vitamin synthesis is associated with young

leaves and transported to the remaining plant (Torrey, 1967).

Plant culture media containing carbohydrates, minerals and vitamins is very nutritional and will support the growth of many unwanted organisms. Therefore, in plant organ culture, as in other types of plant culture both the media and the plant material must be sterile and all transfers and procedures must be done with complete asepsis.

Other plant parts can be cultivated in the same manner as excised roots. Plant apical meristems can be excised and placed on solidified agar medium and may undergo almost unlimited growth without developing roots. Small leaves or leaf primordia may be placed on culture medium and will develop into leaves that attain their characteristic shape. Pollinated flowers may be excised from the plant and will develop whole fruits in vitro (Torrey, 1967).

In each case the successful cultivation allows the discovery of the synthetic capability of the excised part. It must be emphasized that each cultivation of a new species represents a new experimental problem. This problem may be examined by using a procedure that was successful for another species. The growth medium may be modified or a new one may be developed. There is no guarantee that an investigator will be successful in growing a plant organ or plant tissue in vitro.

Callus Tissue Culture

Unorganized plant tissues can also be grown in culture. The result is a proliferating mass or callus which typically possesses no organization and little or no cell differentiation. These masses can be subcultured at suitable intervals.

The major difference in callus tissue culture as compared with

organ culture is the upset of normal hormonal relationship when the tissue is excised from the intact plant. In organ culture the centers of hormone synthesis, meristematic regions, remain but in callus tissue culture they are not present and the hormones and vitamins normally present must be added (Torrey, 1967). Thus hormones such as cytokinins and auxins are often added to the medium. In other cases natural supplements such as "coconut milk" and yeast extract are used but these have the disadvantage of not being completely chemically defined. The use of "coconut milk" has declined since the discovery that a major component of the milk, myo-inositol, can replace the natural extract (Pollard et al., 1961).

Detailed methods for tissue culture have been described by White (1963) and Willmer (1966). Basically these methods involve media preparation, pH adjustment to approximately 5.4 (which is the point that an unbuffered aqueous solution is in equilibrium with the CO_2 of the air), dispensing into 125-ml erlenmeyer flasks which are then sterilized. Tissue explants from the plant are aseptically made and placed on the agar solidified medium. The flasks are then incubated at 25°C and often with a lighting regime. The source of the tissue can vary. White (1963) divided the plant into four types of cells. First there are meristematic regions (which continually grow except for seasonal changes) such as shoots and roots which are routinely grown as organ cultures. The cambium is excised and used for callus culture. Next there are secondary meristems such as cork cambium, ray parenchyma and root pericycles that are capable of intermittent growth but normally do not grow with any continuity. These are generally excised for callus culture. The third group involves differentiated tissue capable of undergoing a

dedifferentiation and becoming meristematic. Thus cells such as cortex parenchyma, and pith cells have been used in callus culture. The last group of cells are living but so highly differentiated that they cannot become meristematic. Cells such as stomata, glandular hairs, and tracheids cannot be used in culture due to their extreme differentiation.

Cell Culture

Cell culture can be accomplished by first growing callus tissue and then breaking the callus culture apart in liquid suspension cultures. The result is a mixture of single cells and small cell groups. The medium and environmental conditions for this type of culture is the same general design as for callus culture.

Chapter 3

MATERIALS AND METHODS

Callus Tissue Culture

The medium of Murashige and Skoog (1962) was chosen as the basic starting medium on which minor modifications would be made until growth was achieved. This medium was selected because of the author's previous success in culturing Nicotiana species (tobacco). In using this medium it was assumed that the basic formula would provide all the necessary inorganic ions for growth and that the successful growth of C. acuminata tissue would depend on supplying the needed vitamins, supplements, auxins and cytokinin in the proportions necessary for cell division and growth. The quantities of inorganic constituents of this medium are given in Table 3. Stock solutions were made so that the quantity of chemical could be delivered volumetrically thus saving time with no loss of accuracy. The combination of chemicals in a stock solution was chosen to minimize any interaction and storage problem. The formula for the four stock solutions is given in Table 3 along with the volumes used to make one liter of medium. After preparing the stock solutions they were sterilized by filtration.[3] The apparatus used is depicted in Figure 4. The liquid to be filtered was forced, with positive pressure, from flask A through filter B and into the sterile flask C from

[3]Millipore Corporation, Bedford, Mass. 0.22 filter.

Table 3. Inorganic Constituents Required for Callus Growth of *C. acuminata*-Showing Quantities for Preparation of Stock Solutions

Chemical[a]	Stock Weight (g)	Stock Solution Number	Stock Volume (ml.)	Volume per L (ml.)	Weight per L
NH_4NO_3	33	1	1000	50	1.7 g
KNO_3	38				1.9 g
$CaCl_2 \cdot 2H_2O$	8.8				0.44 g
$MgSO_4 \cdot 7H_2O$	7.4				0.37 g
KH_2PO_4	4.25	2	250	10	0.17 g
$FeSO_4 \cdot 7H_2O$	1.39	3	250	5	27.8 mg
Na_2EDTA[b]	1.86				37.2 mg
H_3BO_3	0.620	4	1000	10	6.2 mg
$MnSO_4 \cdot 4H_2O$	2.230				28.3 mg 22.3 mg RL
$ZnSO_4 \cdot 4H_2O$	0.860				8.6 mg
KI	0.083				0.83 mg
$Na_2MoO_4 \cdot 2H_2O$	0.025				0.25 mg
$CuSO_4 \cdot 5H_2O$	0.0025				0.025 mg
$CoCl_2 \cdot 6H_2O$	0.0025				0.025 mg

[a]Matheson, Coleman and Bell, Los Angeles, Ca. Reagent Grade.

[b]Ethylenedinitrilo-tetraacetic Acid, Disodium salt.

there it is dispensed into sterile bottles and stored at 4°C.

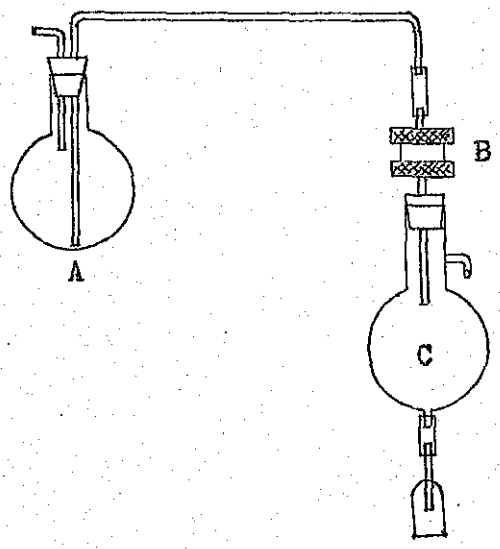


Figure 4. Filtering Apparatus

Organic stock solutions were prepared in the same way, filtered and stored at 4°C. Sucrose, agar, auxin (IAA or 2,4-D), and kinetin were added at the time of medium preparation by weighing (if over 0.5 mg). If smaller quantities of these compounds were needed, a solution was made and an exact volume was dispensed corresponding to the required weight.

Media were prepared as follows: to 670 ml of distilled water were added 50 ml stock solution-1, 10 ml stock solution-2, 5 ml stock solution-3 and 10 ml stock solution-4 (see Table 3). These solutions were combined in a 2-liter beaker and stirred with a magnetic stirrer. Then 250 ml of distilled water were added to 10 g agar and put in a 400-ml beaker and stirred with a glass rod. The contents of the 2-liter beaker were brought to a boil and the agar suspension was added. The mixture was boiled until it became clear. The heat was removed and the 2-liter beaker and contents

were allowed to cool for five minutes and the organic constituents were added either as solids or volumetrically. The solution was stirred thoroughly and the pH adjusted to 5.5 with 0.1N nitric acid or 0.1N sodium hydroxide (pHydrion paper, Microessential Laboratory, Brooklyn, N.Y.). Fifty ml of the solution was then dispensed into 125-ml erlenmeyer flasks, using the approximate calibration lines on the flask. The flasks were then covered tightly with aluminum foil and sterilized at 15 pounds per square inch steam pressure for 15 minutes. After completion of sterilization the flasks were cooled and stored at room temperature until ready for use.

The plant tissues used were derived from two year old branches and seeds removed from fresh C. acuminata (tree K5-6) at the Chico USDA Station. The branches and seeds were collected by USDA Horticulturist R. L. Smith or the author. The seeds were germinated and both the young seedlings and the older branches were used as sources of tissue explants in attempts to produce callus. The explants included cambium sections, older leaf parts, cotyledon leaf parts, pith and stems of young seedlings. It was discovered that only cambial sections and cotyledons would successfully produce callus tissue. The cambium was prepared by the method of White (1963), with minor modifications. A fresh two year old branch was cut into 10 cm lengths, cleansed with tap water for a brief period and then surface sterilized with 95 percent ethanol on cotton. All leaf scars that could harbor bacteria or fungi were removed. The surface was again washed with 95 percent ethanol and the branch section was immersed in sterile tap water. Then the container and branches were taken to the sterile room which employs ultraviolet germicidal lights, antiseptics and bacterially filtered air. The branches were then immersed in either 5 percent sodium

hypochlorite (Clorox®) diluted 1:4 with water or 0.1 percent mercuric chloride with 0.05 ml of a wetting agent (Tween 20®) and left for approximately 5 minutes. The branches were removed and rinsed copiously with sterile tap water and were ready for use. Preparation of the cambial tissue consisted of first removing the epidermis with a scraping motion of a scalpel. While this is not superior to the method of White, it was found to be easier than attempting to peel off the epidermis carefully. With practice the scraping became very easy and little tissue damage below the epidermis resulted. Next, two circular cuts were made between two nodes. These cuts went down to the wood and completely around the branch. Finally a longitudinal cut was made to the wood and the whole section was removed (Figure 5). These sections were then cut into approximately 4 mm X 12 mm rectangles. These rectangles of vascular cambium sandwiched between xylem and phloem were then aseptically transferred to the nutrient medium with the basal end up. It was necessary to orient these tissue pieces as to apical and basal end. To accomplish this orientation the branch was marked prior to the cutting of branch sections since in the case of a short 10 cm section it was almost impossible to tell one end from the other on the basis of diameter. The ends were identified by placing a notch in the shape of an arrow at the basal end of the branch section; this notch was carried through the whole procedure (Figure 6). The notch was removed by making a clean scalpel cut prior to placing the cambial section on the medium.

The flasks and the tissue explants were placed in a plant growth chamber (Sherer-Gillette, Model GEG 25-7) with controlled lighting (4-48 inch fluorescent lamps and 6-incandescent lamps) for 10 hours per 24 hours. The temperature was 25±2°C and the relative humidity was 50 percent.

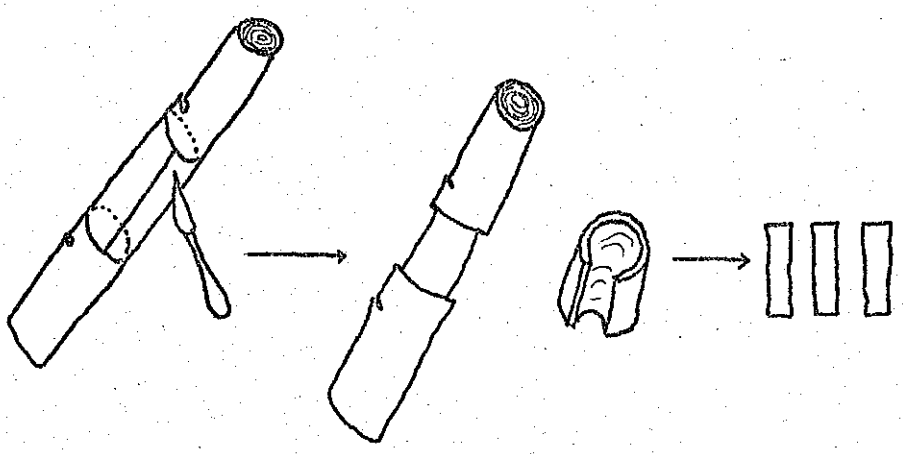


Figure 5. Method of Obtaining Cambial Tissue from a Branch of C. acuminata

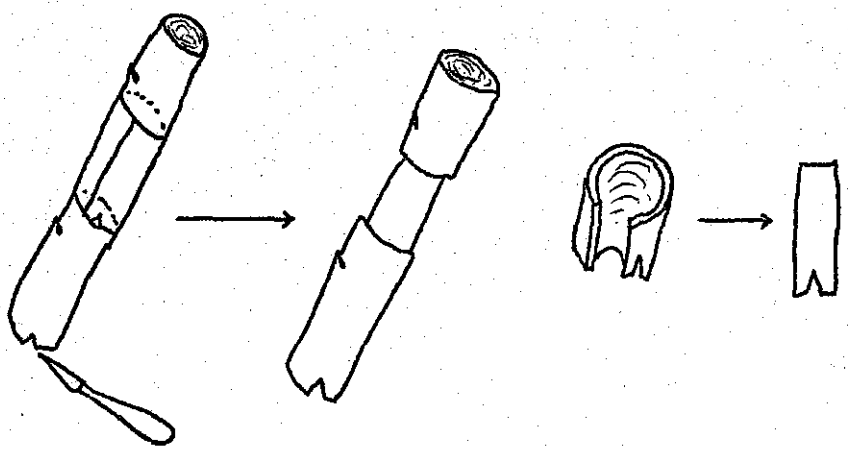


Figure 6. Method of Maintaining Apical/Basal Relationship During Explantation

Seeds from the C. acuminata tree K5-6 were germinated in moistened vermiculite at approximately 21°C. Initially attempts were made to sterilize the seeds and to germinate them in sterile petri dishes on moistened filter paper. Many sterilizing agents were tried: dilute sodium hypochlorite, 0.1 percent mercuric chloride, 1 percent bromine in water and boiling ethanol but all seemed to injure the seed and only a very small percentage would germinate. Therefore the sterilization of seeds prior to germination was discontinued and the seedlings were sterilized after germination in vermiculite using sodium hypochlorite (1:4 with water) or 0.1 percent mercuric chloride followed by thorough rinsing with sterile tap water. The sterile cotyledons were removed from the seedling and placed on the medium. The cotyledons were used either whole (average size approximately 8mm by 30 mm) or cut into halves or quarters.

All tissue transfers were made in a sterile room under a tissue culture hood. Instruments for tissue explantations were sterilized and stored in 70 percent ethanol in the hood; before using, the instruments were flamed, cooled, used in the procedure and returned to the 70 percent ethanol.

Even with these precautions there was contamination--the major source was incomplete plant sterilization. It was found that tissues left in the sterilizing agent for too short a time (e.g. 2 minutes) would be unsterile and those left too long (e.g. 15 minutes) would be sterile but the tissue would be damaged and die after a few days in the medium. Sterility and viability of the tissue could be assured if it was allowed to remain in the sterilizing solution for approximately 5 minutes. This procedure was then adopted.

The initial medium tried was the one used by Murashige and Skoog, (1962) to culture tobacco. The containers were 150-ml screw capped jars. These containers proved to be unsatisfactory. There was no gas exchange and the explanted tissues died quickly. The traditional 125-ml erlenmeyer flask covered with aluminum foil was then used. The same medium was used in these flasks but the tissue did not develop.

In order to maximize the chances of successful callus growth, the inorganic constituents, the sucrose and the agar remained constant in all the media. The ratios of auxin to cytokinin were varied and different vitamins were added. The first ratios of auxin to cytokinin (Table 4) proved to be unsuitable for tissue growth.

Table 4. Auxin to Cytokinin Ratio in mg/liter

IAA	Kinetin
2.0	0.02
2.0	0.2
1.0	0.02
1.0	0.2
0.5	0.02
0.5	0.2

The vitamin pyridoxine hydrochloride was added at 1 mg per liter and the media appearing in Table 4 were again prepared. Once again the tissue failed to develop. Next, the auxin remained at the same 2 mg per liter and the kinetin was tried at concentrations of 0.02, 0.05, 0.1, 0.12, 0.25, 0.5 and 2.0 mg per liter. The tissue once again failed to develop.

Sjolund, (1971) suggested changing the auxin supply from IAA to NAA or 2,4-D, using the regime of Table 5.

Table 5. A Procedure for Increasing the Chances of Callus Growth in 15 Trials

Auxin (mg/L)	Kinetin (mg/L)		
	0.1	0.5	1.0
0.1	1	2	3
0.5	4	5	6
1.0	7	8	9
2.0	10	11	12
2.5	13	14	15

Romberger and Tabor, (1971) noted that bacteriological grade agar when used as a stiffening agent in callus culture could inhibit the tissue growth. Bacteriological grade agar was purified by the following procedure (Corson, 1971): If one liter of a one percent agar solution was required, 500 ml of a two percent solution was made and solidified in a flat pan such that the agar depth was 2.5 cm. The agar was then cut into 2.5 cm cubes and put into a 2-liter beaker and filled with distilled water. The distilled water was changed frequently for the first few hours and thereafter every six hours for two days. The distilled water was poured off and the agar cubes were diluted to one percent when medium was made.

Success was achieved using the basic inorganic constituents (Table 3, p. 30) and the following organic constituents: thiamine

hydrochloride[4]-1 mg, pyridoxine hydrochloride[4] and niacin[4]-0.5 mg each, myo-inositol[4]-100 mg, 2,4-D[5]-2.5 mg, kinetin[4]-0.5 mg, and sucrose[5] 30 g in one liter of medium. Agar purified by distilled water was used at one percent. This medium was used to grow the callus tissue for all experiments. Growth was successful with either water purified agar or commercially purified agar[6].

Cultures of root tips from C. acuminata were attempted many times but always resulted in failure. The procedure was much the same as for obtaining sterile cotyledon tissue. The seeds were germinated in moist vermiculite, the roots excised and sterilized for approximately five minutes in dilute Clorox® or 0.1 percent mercuric chloride. The roots were then rinsed with sterile water and transferred to the liquid culture media in 125-ml flasks. These flasks were incubated at 25°C in the dark and observed periodically for growth. The media employed for root culture was either the basic inorganic constituents (Table 3, p.30) or White's inorganic constituents (White, 1963). To these basic media were added, per liter, niacin-0.5 mg, thiamine hydrochloride and pyridoxine hydrochloride-0.1 mg each, sucrose concentration was 20 g per liter.

Cell suspension culture of C. acuminata was attempted. Several pieces of friable callus were put into four liters of liquid callus medium in an aerated fermenter[7]. The fermenter was kept at 25°C and had

---

[4]Calbiochem, San Diego, Ca.

[5]Matheson, Coleman and Bell, Los Angeles, Ca. (sucrose was reagent grade and 2,4-D was practical grade)

[6]Schwarz-Mann, Van Nuys, Ca.

[7]Virtis Co. Gardiner, N.Y. Series 43 fermenter.

continous agitation. It was noted by microscopic examination after several weeks that the cells were alive but not dividing. It was decided that further modification of the medium would be necessary and cell suspension culture was discontinued.

Extraction and Investigation

Dry C. acuminata branches from the Chico USDA Station[8] were extracted by the method of Wall et al.(1966). The plant material was ground[9] to a fine powder prior to extraction. The powder was defatted for 24 hours with hot hexane in a Soxhlet extraction apparatus and air dried. This air dried marc was similarly extracted with 95 percent ethanol for 24 hours. The ethanolic extract was concentrated in vacuo to dryness. The dried extract was taken up in a small amount of chloroform, filtered through paper and dried over anhydrous sodium sulfate.

The general alkaloid extraction method of Farnsworth, (1961) was also investigated. The ground plant material was wetted with 28 percent ammonium hydroxide, air dried overnight and extracted continuously with hot chloroform in a Soxhlet apparatus for 24 hours. The chloroform extract was evaporated to dryness and the dried residue then extracted with 6 percent acetic acid and filtered through paper. The filtered acid solution was made basic by the addition of 28 percent ammonium hydroxide and extracted three times with chloroform in a separatory

---

[8]Courtesy of horticulturist R.L. Smith.

[9]Wiley Mill, model 3, A.H. Thomas Co., Philadelphia, Pa.

funnel. The chloroform extract was dried over anhydrous sodium sulfate and filtered through paper. The dried chloroform extract should contain most alkaloids. This is a classical method for alkaloid extraction but camptothecin has an unusually reactive lactone group in the molecule and in the presence of base is converted to a salt (Figure 2, p.7). Camptothecin and its analogs (as the ammonium salts) remained in the aqueous layer and thus were not extracted in the chloroform phase. This procedure was found to be unsuccessful.

C. acuminata callus was collected by either removing the entire callus mass from the flask or dissecting a portion of the callus under aseptic conditions and replacing the flask in the environmental chamber. All callus removed from the flasks were retained and the wet weight recorded. The harvested callus was then dried over silica gel and the dry weight recorded. The dried callus was pulverized (mortar and pestle) and extracted by the method of Wall et al. (1966) as described earlier, but it was found that a cold extraction with chloroform in a 5-ml Microflex[10] tube would serve the same purpose in a much shorter time. It was also found that wet callus could be extracted with chloroform in a Microflex tube with the same result.

After the extraction procedure, the chloroform extract of the wood and callus were concentrated to one ml or less in a stream of air and evaluated using thin-layer chromatography (TLC). TLC plates[11] coated with 0.25 mm silica gel G as the adsorbant. The plates were dried at 105°C for one hour and dessicated. The volume of chloroform extract

---

[10] Kontes Glass Co., Vineland, N.J.

[11] Precoated. Brinkman Instruments, Inc. Westburg, N.Y.

(of wood and callus) varied but was in the range of 2-10 µl. Standard solutions of camptothecin[12] in chloroform were made at the following concentrations (mg/ml): 0.04, 0.008, 0.0016, 0.00016 and 0.000016. Standard solutions of hydroxycamptothecin[13] and methoxycamptothecin[13] in chloroform were made at 0.001 mg per milliliter. All TLC evaluated extracts were spotted along with a reference camptothecin, hydroxy-camptothecin and methoxycamptothecin standard solution.

All TLC development was done with two solvent systems: chloroform-acetone-methanol (7.5:2.0:0.5) as used by Smith (1971) at the Chico USDA Station and benzene-acetone-methanol (18:2.0:0.5) as described by Wani and Wall (1969). The solvent was allowed to rise to a scribed 10 cm height on the silica gel plate. The plate was removed from the solvent tank, dried and inspected under long wave ultraviolet (UV) light. The position (Rf value), fluorescent color and relative intensity under UV light was noted. Selected fluorescent spots that had been separated by TLC were removed by scraping off the silica gel. The silica gel containing the compound was extracted with hot 95 percent ethanol in a small flask and the solution filtered through a sintered glass crucible, porosity F, to remove all silica gel. The 95 percent ethanol solution was evaporated to dryness and taken up in solvents such as chloroform, dioxane and 95 percent ethanol for scanning U.V. spectroscopy[14].

---

[12]Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. Courtesy of Dr. Harry Wood Jr.

[13]Research Triangle Institute, Research Triangle Park, N.C. Courtesy of Dr. M.C. Wani.

[14]Perkin-Elmer Model 202.

An estimation of the amount of camptothecin in a typical callus extract was made by visual inspection of the relative fluorescence of the extract as compared to several camptothecin standards after TLC separation.

A cold chloroform extract was made of the medium on which the callus grew. This extract was made during many stages of development: immediately after explantation, after 1 month growth, after 2 month growth and after 5 month growth. A similar extraction of medium which had never supported growth was made. The extractions were accomplished by homogenization of the agar media with chloroform in a blender and draining, filtering and drying of the chloroform over sodium sulfate. The dried chloroform extract was concentrated to under 1 ml volume and subjected to TLC evaluation.

After individual TLC evaluation of 70 samples of friable and compact callus, the cold chloroform extract of the 70 samples was pooled and evaporated to dryness. The resultant crude extract (100 mg) was amorphous and yellow in color. The crude extract was taken up in a minimum volume of warm benzene-acetone-methanol mixture (18:2.0:0.5) and applied to a chromaflex[15] column (25 cm x 2.5 cm) of silica gel G for column chromatography[16] (12 cm of silica gel). The column had been wet packed with the silica gel in the benzene-acetone-methanol mixture. The extract was eluted with the same mixture. The fluorescent bands were observed to separate and as the first band came out, 3-ml fractions were collected in 5-ml test tubes with an automatic fraction collector[17].

---

[15] Konte Glass Co. Vineland N.J.

[16] E. Merck, Germany.

[17] Gilson Co. Distributed by West Coast Scientific, Los Angeles, Ca.

Twenty fractions were collected and all were investigated for purity of individual fractions by TLC methods. Selected fractions that were shown to consist of one fraction by TLC analysis were subjected to other examinations. These fractions consisted of white amorphous solids of much less than 1 mg. The fractions were subjected to UV spectroscopy (95 percent ethanol) and solubility test in 2N hydrochloric acid. They were also tested with non-specific alkaloidal reagents (Mayer's, Phosphotungstic Acid, Wagner's).

Photographs were taken of callus in various stages of growth, from initial implantation to repeated subculture. Compact callus was imbedded in paraffin by standard methods and sectioned at 8 microns. The sections were stained using a safranin-fast green combination to stain cell walls green and nuclear material red. The sectioning and staining techniques are those of Jensen (1962). Permanent mounts of compact callus were made and photomicrographs taken. Friable callus could not be imbedded in paraffin by standard means because of its fragile nature and thus was observed and photographed through the microscope as whole mounts in water.

Chapter 4

RESULTS

As previously stated the media were varied in a systematic fashion and ultimately a medium was found that would induce callus tissue to form. This medium consisted of the inorganic ions plus the organic constituents. (Table 6)

Table 6. Organic Constituents of Medium used for Induction and Growth of C. acuminata Callus

Constituent	Weight per Liter
Sucrose	30g
Agar	10g
myo-Inositol	100mg
2,4-D	2.5mg
Kinetin	0.5mg
Thiamine-HCl	1.0mg
Pyridoxine-HCl	0.5mg
Niacin	0.5mg

The cambium sections of C. acuminata (Figure 7) were observed daily for growth. Callus growth was evident after 7 days. The callus appeared as white, crystalline outgrowths from the cambium at the agar level (Figure 8). It was observed that sometimes growth would start but then unexplainably die; the result was a very dark mass (Figure 8). The viable callus was allowed to grow to a large size (30-60 days) and

Figure 7. Cambial Sections Obtained from C. acuminata Branch and Placed On Nutrient Medium at Day 0

Figure 8. Callus Growth from Cambial Sections after Day 20

then very carefully separated from any original tissue and subcultured on the same medium. These subcultures grew very large over a few months time and gave rise to masses with various colors (Figure 9). Usually the healthy white tissue was found near the edges and the middle was of a dull brown color and slightly necrotic. The callus was found to be of a compact nature. Thin sections revealed a typical callus morphology: large cells in the central regions with very dilute cytoplasm and smaller, meristematic cells located on the periphery with obvious nuclei (Figure 10).

The cotyledon cultures were examined daily for growth (Figure 11). Callus growth was noted in about 7 days. The callus appeared as white growths at the margins of the leaf (Figure 12). Eventually the growth would become so large that the original cotyledon tissue was obscured (Figure 13). The cotyledon callus was subcultured on similar medium. The callus was of a friable nature and subculture consisted of removing a section with a sterile spatula and transferring to a fresh flask. Whole mounts in water of the mushy grey or brown callus showed in some cases a typical plant cell (Figure 14), with cell wall, nucleus, and observable cytoplasmic streaming. In other cases very strange cells and cell aggregates could be observed (Figure 15). Cells ranged from small to very large. All cells observed were comparable to observations made of suspension cultures as noted in Willmer (1966).

After an accident with the environmental chamber in which all cultures were frozen, approximately one quarter of all newly started cultures (cotyledon origin) were left outside the chamber. These were noted to be greyish. Cultures left in the incubator were noted to have very slight red pigmentation. With an increase in the number of hours of

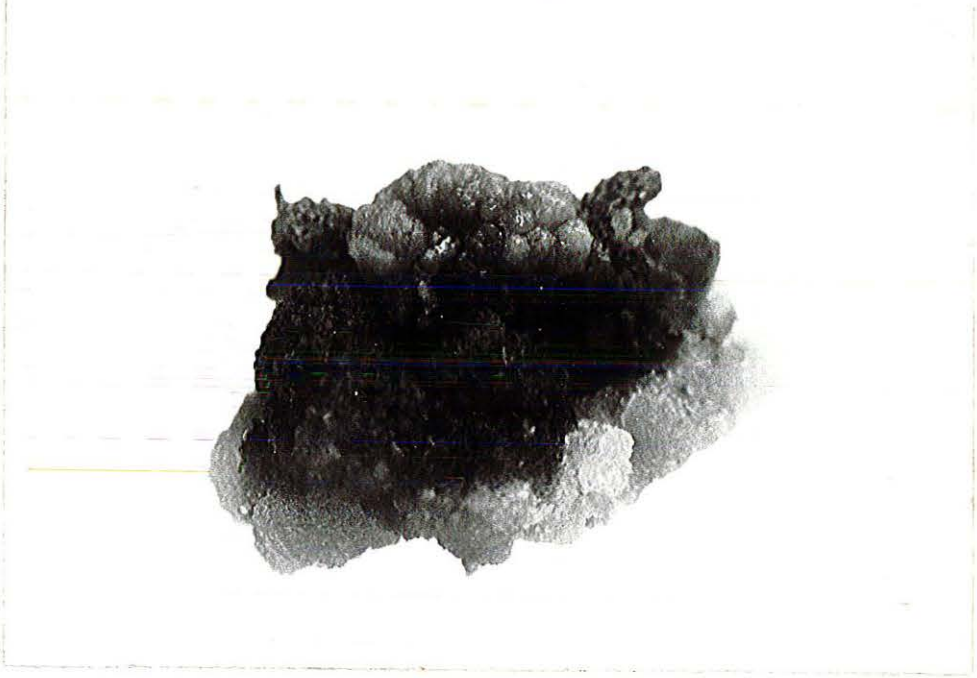


Figure 9. Subcultured Callus from Cambial Sections of C. acuminata

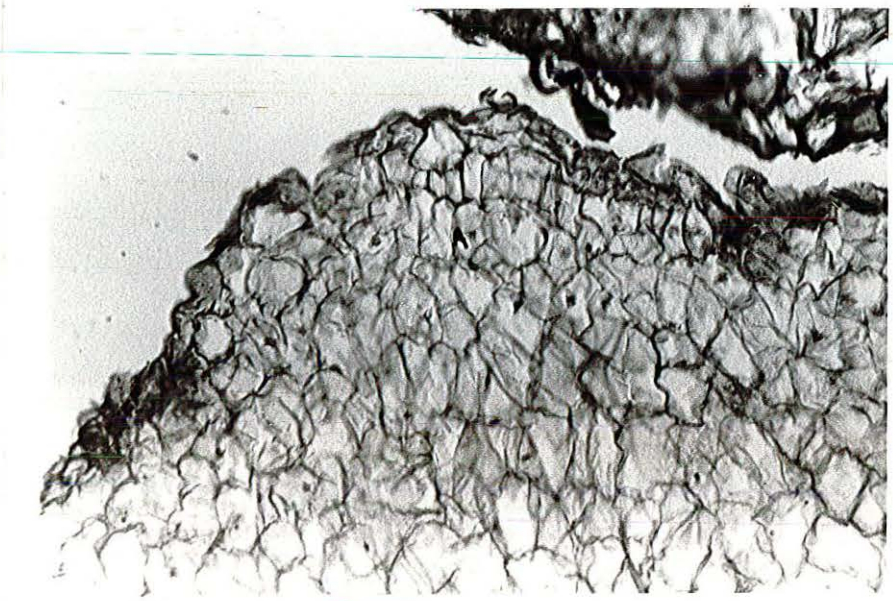


Figure 10. Cross Sections of Callus from the Cambium of C. acuminata X100

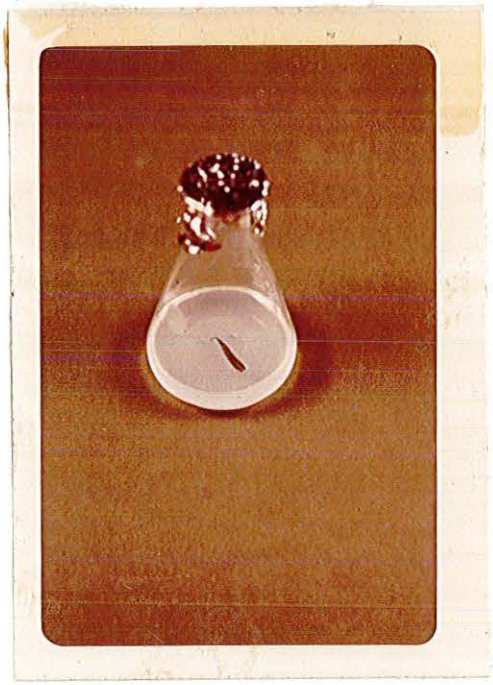


Figure 11. Cotyledon of C. acuminata Placed on Callus Medium at Day 0

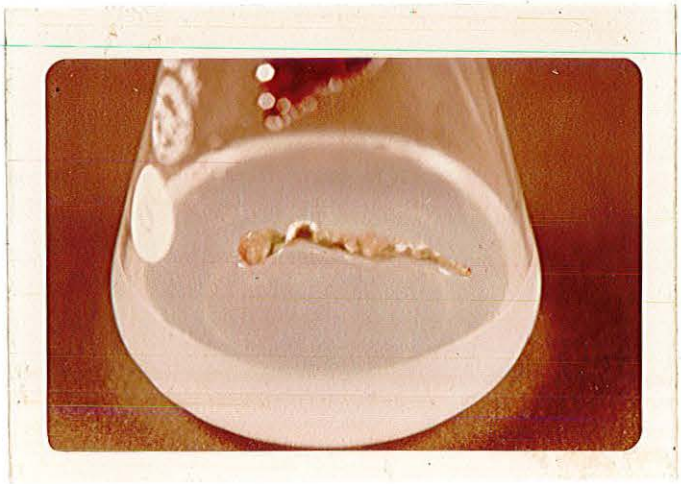


Figure 12. Cotyledon of C. acuminata Showing Callus Outgrowths at the Margins-Day 15

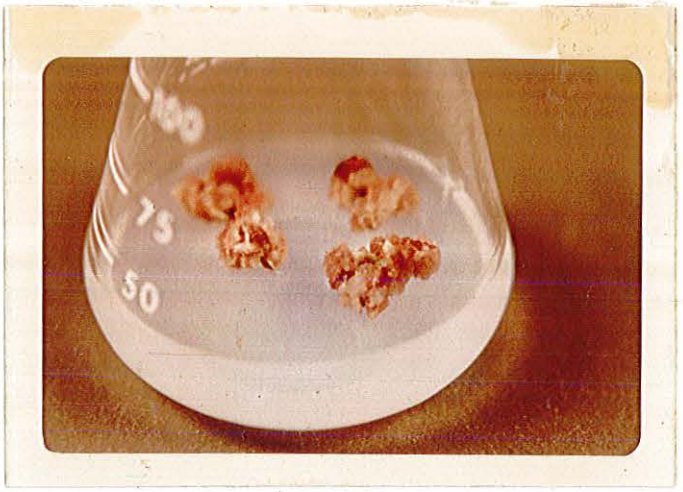


Figure 13. Callus Tissue Obscuring the Cotyledons of C. acuminata-Day 45

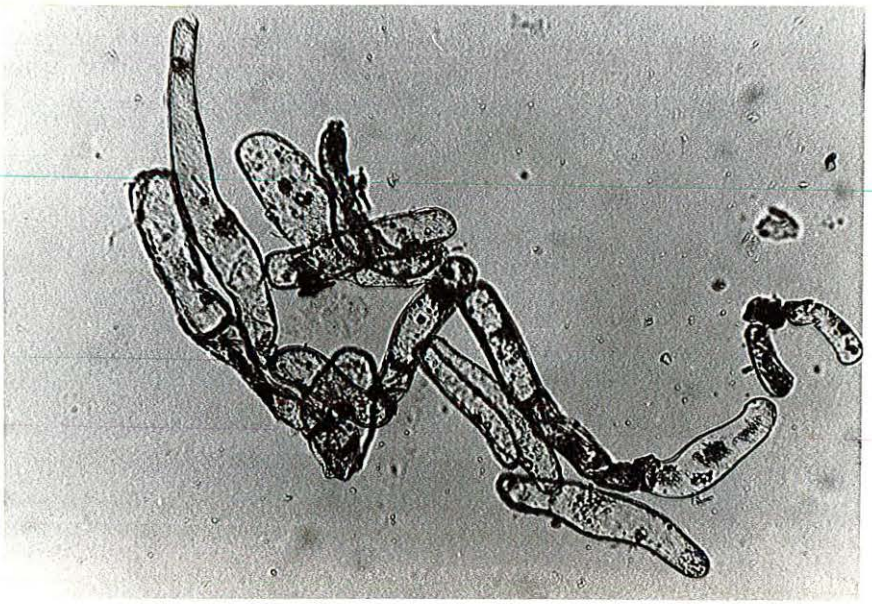


Figure 14. Whole Mount of Friable Callus Tissue Obtained from Cotyledons of C. acuminata X100

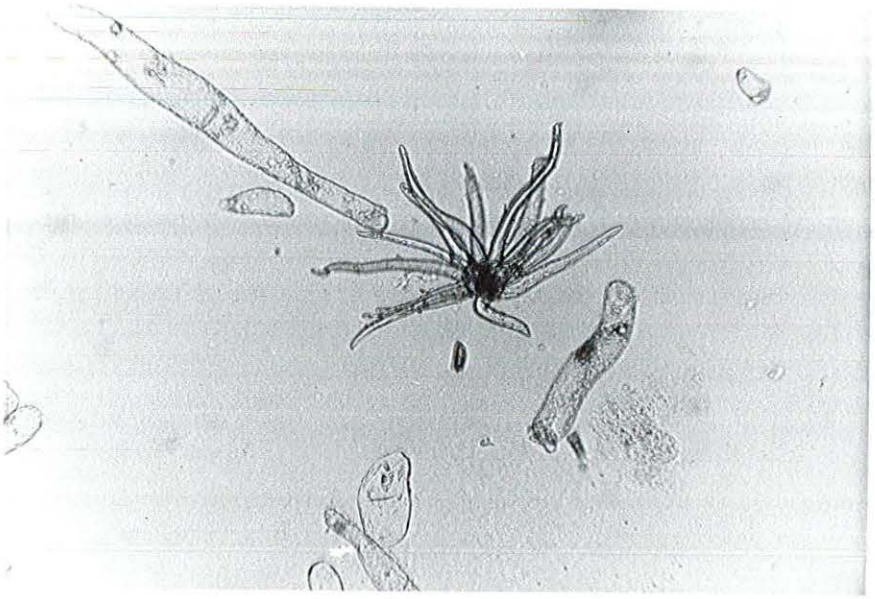


Figure 15. Whole Mount of Friable Callus Showing Typical Cells and Atypical Cell Aggregation X100

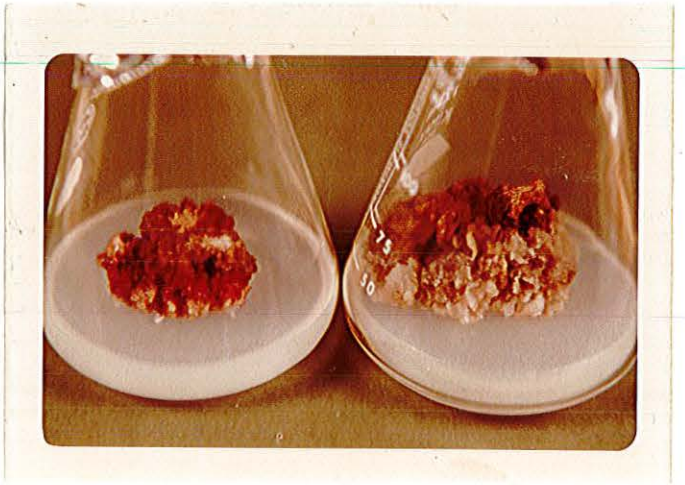


Figure 16. Callus Tissue (Cotyledon Origin) Observations with Different Lighting Conditions--the Callus On the Left has been Exposed to Intense Lighting and the Callus on the Right has been Exposed to Normal Room Lighting

light per day (16) a very noticeable red pigmentation appeared. A comparison of these two is shown in Figure 16.

Camptothecin was shown to be present in initial callus outgrowths from explants. Eleven day old callus was observed to have camptothecin (R_f 0.55 in chloroform-acetone-methanol (7.5:2.0:0.5) comparable R_f value to standard and comparable UV spectra). Other analytical tests were precluded due to minute quantities. Callus of 17 days was observed to have camptothecin plus two other unknown compounds R_f 0.95 and 0.85 as visualized under long wave UV light. A visual estimation gave the camptothecin concentration in these tissues an approximate value of 0.02 μg per mg dry weight of callus which is 0.002 percent on the dry weight basis. After thirty days camptothecin had disappeared and the two above compounds predominated. It was found that the explants in close proximity to the developing callus that were analyzed had camptothecin at day 11, 17, 30 and beyond. It was found that both camptothecin and the other two chemicals with higher R_f values diffused into the agar and all 3 were detected in the agar for as long a period as 150 days. A chloroform extract of media which had not supported growth was observed to have a slight fluoresence at the baseline.

Callus subcultured one or more times and the medium on which it developed contained the two unknown compounds with R_f values of 0.95 and 0.85.

The callus tissue pooled from 70 flasks of both tissue origins was examined. The wet weight varied from 15.0 g (many pieces grown on 400 ml medium in a one liter flask) to 0.129 g. The dry weight of these two was 1.058 g and 0.008 g, respectively. The usual water content

of the callus was 90 percent and above. TLC data on the 70 samples showed the two fluorescent areas at R_f 0.95 and 0.85 and occasionally on concentration of the chloroform extract two additional fluorescent compounds were noted under UV light. The location of these two compounds were at R_f 0.2 and 0.06. These were considered insignificant. In no case did camptothecin appear in TLC evaluation of the 70 samples. The total dry weight of the 70 samples when pooled was 5.4 g. The chloroform extract of the pooled samples yielded 100 mg of an amorphous yellow substance.

An initial extract of C. acuminata branches when evaluated with TLC procedures showed the presence of camptothecin, hydroxycamptothecin, methoxycamptothecin and other unknown fluorescent areas as visualized under UV light but none corresponding to those at R_f 0.95 and 0.85 as seen in the callus extracts.

A later TLC examination in which the extract of the branches was concentrated (exact concentration unknown) to show very faint fluorescent areas at R_f 0.95 and 0.85 corresponding to those found in the callus extract. This result prompted a reevaluation of work done. An additional extract of 50 g wood was made. The 95 percent ethanol extract was evaporated to dryness and taken up in chloroform and concentrated to 1 ml volume. This concentrated extract was compared to the callus extract using TLC procedures (Table 7).

A change of solvent systems for TLC evaluation gave even more surprising data. The spot at R_f 0.95 although very symmetrical throughout a 10 cm solvent movement, was shown to be composed of three fluorescent compounds. The new system was benzene-acetone-methanol (18:2:0.5). Again the wood and callus extracts were chromatographed

with the new solvent system (Table 8).

Table 7. TLC Evaluated Data for C. acuminata Callus and Wood Extracted with Chloroform and 95 percent Ethanol Respectively. The Solvent System was Chloroform, Acetone, Methanol 7.5:2.0:0.5

Tissue	R_f visualized under UV light					
Wood	0.99[a]	0.95	0.85	0.72	0.55[b]	0.32[c]
Callus		0.95	0.85			

[a]Red color, probably a chlorophyll

[b]Large blue oval-camptothecin plus methoxycamptothecin

[c]Hydroxycamptothecin

Column chromatography of the pooled callus extract produced 20 fractions. Collected fractions were investigated for purity of individual compounds by TLC procedures. Fractions 1, 7, 10 and 20 contained a small amount of the four fluorescent unknown compounds (Table 8). All were white compounds and all were present in quantities less than 1 mg. It was found that these four purified compounds were insoluble in 2N HCl and would not precipitate alkaloid reagents (Mayer's, Wagner's, Silicotungstic acid). It was noted that in TLC procedures the four compounds were visualized by UV light but could not be visualized by alkaloidal spray reagents (Dragendorff Reagent, Iodoplatinate). The UV spectra of the four compounds gave the following data: UV_{max} of 210, 230 sh, 280 and 285 sh nm for all four fluorescent spots.

Table 8. TLC Evaluated Data for Callus and Wood Extracted with Chloroform and 95 percent Ethanol Respectively-Solvent System was Benzene, Acetone, Methanol 18:2:0.5

Tissue	R_f	Color under U.V. (long wave)	Relative intensity
Callus	0.67	Blue	++
	0.60	Blue	+++
	0.51	Blue	++
	0.36	Grey-Blue	+
Wood	0.93	Red[a]	++
	0.67	Blue	+
	0.60	Blue	+
	0.51	Blue	++
	0.36	Blue	+
	0.26	Blue	+
	0.21	Yellow	+
	0.17	Blue[b]	++
	0.14	Blue[c]	++++
	0.09	Blue[d]	++++
	0.03	Violet	+++

[a] Probably chlorophyll

[b] Camptothecin

[c] Methoxycamptothecin

[d] Hydroxycamptothecin

Chapter 5

DISCUSSION

Tissue cultures of medicinal plants have been grown and active compounds from these cultures are reported. Examples are the production of alkaloids in tissue cultures of Datura (Chan and Staba, 1965) and antimicrobial substances (Khanna and Staba, 1968). The plants used in these cultures were not in limited supply. Camptotheca acuminata offered a rather unique situation in that the plants were in short supply and in great anticipated demand due to the excellent antitumor activity of its extracts observed in preliminary investigations. Initially the chemical principle was unknown and its synthesis in sufficient quantity, even if it were known, was unpredictable.

The tissue culture of C. acuminata was an attractive alternative in view of the short supply of the live plant. If the tissue cultures yielded the active principle(s) in sufficient quantity, this in vitro method of growth would be expected to be less expensive and assure adequate supply under controlled conditions of growth in a shorter period of time.

Investigations of this alternative involved two steps. First to establish if C. acuminata tissues could be grown in vitro at all and second to establish if, after tissues are grown in vitro, the active principle would be synthesized by the tissues under these "unnatural" conditions. After these two steps were firmly established, scale-up to yield larger quantities of active principle could be attempted.

In order to investigate the possibilities of establishing tissue

cultures of C. acuminata the inorganic and organic nutrients, hormonal and environmental factors had to be considered. The medium for tissue culture growth was selected on the basis of its previously reported success in culture of higher plants. It was realized that slight modifications would be necessary for the culture of C. acuminata tissues.

The ratio of cytokinin/auxin is the most important variant in the successful growth of a plant tissue under culture. It was noted in this investigation that 2,4-D was superior to IAA or NAA for callus induction of C. acuminata. Wetmore and Rier (1963) also have made this observation with other species. It has been observed by Sjolund (1971) that 2,4-D is more stable than IAA in culture media.

The cytokinin/auxin ration has to be experimentally established for each plant under investigation. Willmer (1966) has given guideline ranges of variance of auxin between 0.01 to 10 mg/L and cytokinin between 0.1 to 10 mg/L of medium. The variance of auxin and cytokinin suggested by Sjolund (1971) is seen in Table 5 (p. 37). It can be observed that a ratio of 2.5 mg of 2,4-D to 0.5 mg of kinetin gave best growth for C. acuminata in the experiment.

Cambium was chosen for an explant because of its meristematic nature. The cambial rectangles were positioned with the apical end submerged as described by White (1963), to take advantage of the normal polar movement of auxin from the apical to the basal end. The orientation of basal end up, with the subsequent accumulation of auxin due to polar movement from apical to basal end was conducive to callus formation.

The choice of cotyledons for a source of callus was somewhat unusual. The leaf contains meristematic regions and partially differentiated cells capable of dedifferentiation and was therefore a

candidate for a tissue source.

The observation of two types of callus (friable and compact) from the two sources of tissue may be unique. It has been noted (Willmer, 1966) that transitions from friable to compact callus and vise versa, occur with a change in the medium formulation, notably in the auxin concentration. In the case of C. acuminata the medium is the same and only tissue source differs.

Romberger and Tabor (1971) have reported that the use of unpurified agar in solid culture media may be inhibitory for some callus culture. In the present series of experiments on C. acuminata purification of agar was not shown to be an absolute requirement. However in one case an accidental substitution of unpurified agar occurred and cambial explants were placed on the medium. It was observed that more sections failed to produce callus in this case than on medium prepared with purified agar and also the growth of callus, when occurring, was slower.

The production of a red pigment in callus tissue of C. acuminata grown under intense lighting conditions (16 hours per 24 hours) may be due to synthesis of an anthocyanin pigment. However no attempts were made to prove this point in the experiments reported here. It has been reported by Ibrahim et al.(1971) that anthocyanin production in callus tissue is rare and in vitro synthesis may be controlled by illumination, presence and amount of auxin or other growth factors.

The extraction and isolation procedures, R_f value on TLC, color of spots under long wave UV light and UV absorption spectra of the callus extract after 11 days of culture indicated fairly conclusively that camptothecin was present. This evidence is not however absolute. After

30 days of culture there was no indication of the presence of camptothecin in the callus growth. It would be possible that camptothecin present in the cotyledon would diffuse into the newly grown, 11 day callus under the solid culture conditions. However, careful excision of the callus and cotyledon in close proximity to each other after 11 day and 30 day cultures and TLC evaluation indicated that camptothecin was present in the cotyledon in both cases and was absent in the 30 day callus. If diffusion of camptothecin from the original cotyledon to the callus were to take place one would expect its presence in both 11 day and 30 day callus.

The above observation indicated that the callus cells lose their ability to synthesize camptothecin in a period of 30 days. In TLC evaluation four other fluorescent spots were observed (Table 8, p.54). These do not appear to be structurally related to camptothecin. It could be possible that the four fluorescent spots would be metabolites of camptothecin. Their mobilities on silica gel TLC plates, insolubility in dilute HCl and failure to give any positive reaction with alkaloidal reagents indicates that they are non-alkaloidal in nature. The UV spectra also shows that the four compounds do not have the camptothecin chromophore. It is most unlikely that these four spots are due to metabolic breakdown of camptothecin in the callus tissue since they are also observed to be present in the original plant extracts and even minute quantities of camptothecin (0.016μg/ml) in equilibrium with breakdown products would be sufficient to show up in the TLC procedures utilized.

These observations showed that under the conditions employed in these reported experiments the tissue culture of Camptotheca acuminata did not produce significant quantities of camptothecin. Further

modification of medium, growth factors and growth conditions may be successful in the second part of the project. This will require further investigation.

Chapter 6

SUMMARY

Camptotheca acuminata Decaisne (Nyssaceae) is the source of the potential anticancer compound camptothecin. The supply of the plant in the United States is limited. Callus tissue of the plant was grown *in vitro*. The sources of the tissue for the culture were from the cambium and cotyledon resulting in a compact and friable callus respectively. Callus from both sources were found to contain camptothecin in a very small amount for the first two weeks in culture. Beyond this time four other unknown fluorescent compounds predominated and camptothecin was completely absent after 30 days. TLC derived data, UV spectral data, solubility properties and chemical data suggested that these compounds were non-alkaloidal and not related to camptothecin.

LITERATURE CITED

Audus, L.A. 1959. Plant Growth Substances. Interscience Publishers Inc. N.Y. p. 372.

Bosmann, H.B. 1970. Camptothecin Inhibits Macromolecular Synthesis of E. coli. Biochem. and Biophys. Res. Comm. 41:1412.

Bergmann, L. 1960. Growth and Division of Single Cells of Higher Plants in Culture. J. Gen. Physiol. 43:841.

Cancer Chemotherapy National Service Center. 1962. Protocols for Screening Chemical Agents and Natural Products against Animal Tumors and other Biological Systems. Cancer Chemother. Rep. 25:1-65.

Chan, W.N. and E.J. Staba. 1965. Alkaloid Production by Datura Callus and Suspension Tissue Cultures. Lloydia 28:55.

Corson, G.E. 1971. Personal Communication. Dept. of Biological Sciences, Chico State College, Chico, Ca.

Farnsworth, N.R. 1961. The Pharmacognosy of the Periwinkles: Vinca and Catharanthus. Lloydia 24:105.

Gautheret, R.J. 1934. Culture du Tissu Cambial. C.R. Acad. Sci. Paris 198:2195 in Willmer, E.N. (Ed.) 1966. Cells and Tissues in Culture, Vol. 3. Academic Press, N.Y., N.Y. p. 462.

Gottlieb, J.A., A.M. Guarino, J.B. Call, V.T. Olivero and J.B. Block. Preliminary Pharmacologic and Clinical Evaluation of Camptothecin Sodium. Cancer Chemother. Rep. 54:461.

Haberlandt, G. 1902. Kuturversuche mit Isolierten Pflanzenzellen. Sitzungsber Akad. der Wiss. Wien, Math. Naturwiss. Kl. 3:69 in White, P.R. 1963. The Cultivation of Animal and Plant Cells 2nd. Ed. The Ronald Press Co. N.Y. p. 8.

Harrison, R.G. 1907. Observations on the Living Developing Nerve Fiber. Proc. Soc. Exp. Biol. 4:140 in Paul, J. 1970. Cell and Tissue Culture. 4th Ed. The Williams and Wilkins Co., Baltimore, Md. p. 1.

Hart, L.G., J.B. Call and V.T. Oliverio. 1969. A Fluorimetric Method for Determination of Camptothecin in Plasma and Urine. Cancer Chemother. Rep. 53:211.

Horwitz, S.B., C. Chang and A.P. Grollman. 1970. Mechanism of Action of Camptothecin. The Pharmacologist 12:283.

Ibrahim, R.K., M.L. Thakur and B. Permanand. 1971. Formation of Anthocyanins in Callus Tissue Culture. Lloydia 34:175.

Jablonski, J.R. and F. Skoog. 1954. Cell Enlargement and Cell Division in Excised Tobacco Pith Tissue. Physiol. Plant. 7:16.

Jacob, F. and J. Monod. 1961. Genetic Regulatory Mechanisms in the Synthesis of Proteins. J. Mol. Biol. 3:318.

Jensen, W.A. 1962. Botanical Histochemistry. W.H. Freeman and Co. San Francisco, Ca.

Kessel, D. 1971a. Effects of Camptothecin on RNA Synthesis in Leukemia L-1210 Cells. Biochim. Biophys. Acta. 246:225.

_____. 1971b. Effects of Camptothecin on Macromolecular Synthesis by Murine Leukemia Cells. Proc. Amer. Assoc. Cancer Res. 12:4.

Khanna, P. and E.J. Staba. 1968. Antimicrobials from Plant Tissue Cultures. Lloydia 31:180.

Kotte, W. 1922. Wurlzemeristem in Gewebekultur. Ber. Deut. Bot. Ges. 40: 269 in White, P.R. 1963. The Cultivation of Animal and Plant Cells 2nd Ed. The Ronald Press Co. N.Y. p. 15.

Miller, C.O., F. Skoog, F.S. Okumura, M.H. vonSalza and F.M. Strong. 1955. Structure and Synthesis of Kinetin. J.A.C.S. 77:2662.

Moore, B.G., H.S. Schwartz and H.Hodo. 1970. Inhibition of Macromolecular Synthesis in L-1210 Ascites Tumor Cells by Camptothecin. J. Cell Biol. 47:144a.

Muir, W.H., A.C. Hildebrant and A.J. Riker. 1954. Plant Tissue Cultures Produced from a Single Isolated Cell. Science 119:877.

Murashige, T. and F. Skoog. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 15:473-479.

Nobecourt, P. 1937. Culture en Serie de Tissus Vegetaux sur Milieu Artificiel. C.R. Acad. Sci. Paris 205:521 in Willmer, E.N. (Ed.) 1966. Cells and Tissues in Culture Vol. 3. Academic Press, N.Y., N.Y. p. 462.

Paleg, L.G. 1960. Physiological Effects of Gibberellic Acid I. Carbohydrate Metabolism and Amylase Activity in Barley Endosperm. Plant Physiol. 35:293.

Perdue, R.E. 1968. Camptotheca acuminata-Source of Promising Cancer Drug. Lasca Leaves September:55.

_____, M.E. Wall, J.L. Hartwell and B.J. Abbott. 1968. Comparison of the Activity of Crude Camptotheca acuminata Ethanolic Extracts Against Lymphoid Leukemia L-1210. Lloydia 31:229.

Pollard, J.K., E.M. Shantz and F.C. Steward. 1961. Hexitols in Coconut Milk Their Role in Nurture of Dividing Cells. Plant Physiol. 36:492.

Rinert, J. 1956. Dissolution of Cultures of Picea glauca into Small Tissue Fragments and Single Cells. Science 123:457.

Robbins, W.J. 1922. Cultivation of Excised Root Tips Under Sterile Conditions. Bot. Gaz. 73:376.

Romberger, J.A. and C.A. Tabor. 1971. The Picea abies Shoot Apical Meristem in Culture. I. Agar and Autoclaving Effects. Amer. J. Bot. 58:131.

Salisbury, F.B. and C. Ross. 1969. Plant Physiology. Wadsworth Publishing Co., Belmont, Ca.

Sjolund, R.D. 1971. Personal Communication. Dept. of Botany, University of Iowa, Iowa City, Iowa.

Skoog, F. and C.O. Miller. 1957. Chemical Regulation of Growth and Organ Function in Plant Tissues Cultured in vitro. The Biological Action of Growth Substances. Symp Soc. Exp. Biol. 11:118 in Torrey, J.G. 1967. Development in Flowering Plants. McMillan Co. London. p. 19.

Smith, R.L. 1971. Personal Communication. USDA Plant Introduction Station. Chico, Ca.

Stork, G. and A.G. Schultz. 1971. The Total Synthesis of dl-Camptothecin. J.A.C.S. 93:4074

Torrey, J.G. 1967. Development in Flowering Plants. McMillan Co. London.

Volkmann, R., S. Danishefky, J. eggler and D.M. Solomon. 1971. A Total Synthesis of dl-Camptothecin. J.A.C.S. 93:5576.

Wagner, H. and L. Horhammer (Eds.). 1971. Pharmacognosy and Phytochemistry. Springer-Verlag, N.Y.

Wall, M.E., M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail and G.A. Sim. 1966. Plant Antitumor Agents I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca acuminata. J.A.C.S. 88:3888.

Wani, M.C. and M.E. Wall. 1969. Plant Antitumor Agents II. The Structure of two New Alkaloids from Camptotheca acuminata. J. Org. Chem. 34:1364.

_____, H.F. Campbell, G.A. Brine, J.A. Kepler, M.E. Wall and S.G. Levine. 1972. Plant Antitumor Agents IX. The Total Synthesis of dl-Camptothecin. J.A.C.S. 94:3631.

Wetmore, R.H. and J.P. Rier. 1963. Experimental Induction of Vascular Tissues in Callus of Angiosperms. Amer. J. Bot. 50:418.

White, P.R. 1934. Potentially Unlimited Growth of Excised Tomato Root Tips in a Liquid Medium. Plant Physiol. 9:585.

White, P.R. 1939. Potentially Unlimited Growth of Excised Plant Callus in an Artificial Nutrient. Amer. J. Bot. 26:59.

_____. 1963. The Cultivation of Animal and Plant Cells 2nd Ed. The Ronald Press Co., N.Y.

Willmer, E.N. (Ed.) 1966. Cells and Tissues in Culture. Vol. 3. Academic Press. N.Y., N.Y.

Wood, H.B. 1971. Personal Communication. National Cancer Institute. Chief, Drug Development Branch, Drug Research and Development.